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(54) Title: USE OF 7 ALPHA-SUBSTITUTED STEROIDS TO TREAT NEUROPSYCHIATRIC, IMMUNE OR ENDOCRINE DISORDERS

(57) Abstract

Use is provided for a 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or an analogue thereof substituted independently at one or both of the 7- and 3-positions with an ester or ether group, in the manufacture of a pharmaceutical composition for the therapy of neuropsychiatric, immune and/or endocrine disorders or for inducing cognitive enhancement. Uses for Cyp7b enzymes in producing such steroids is also provided together with various novel steroids and test kits and methods for diagnosing the disorders.

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USE OF 7 ALPHA-SUBSTITUTED STEROIDS TO TREAT NEUROPSYCHIATRIC, IMMUNE OR ENDOCRINE DISORDERS

The present invention relates to novel uses for 7 α -hydroxy-substituted steroids, to a process for preparing such steroids and to novel steroids so produced.

In particular the invention relates to the use of cytochromes of the cytochrome
5 P450 family designated Cyp7b to effect 7 α -hydroxylation of certain 3 β -OH steroids so as to produce a 7 α -hydroxy-substituted steroids. Certain of the 7 α -hydroxy-substituted steroids so produced, as well the corresponding 7-oxo derivatives, are novel and form further aspects of the invention. The invention also relates to uses of these steroids, to uses of Cyp7b enzymes and to uses of novel macromolecular species, eg. antibodies and DNAs,
10 which are biologically related to the Cyp7b enzymes.

Cytochromes P450 are a diverse group of heme-containing mono-oxygenases (termed CYP's; see Nelson *et al.*, DNA Cell Biol. (1993) 12, 1-51) that catalyse a variety of oxidative conversions, notably of steroids but also of fatty acids and xenobiotics. While CYP's are most abundantly expressed in the testis, ovary, placenta, adrenal and liver, it is
15 becoming clear that the brain is a further site of CYP expression. Several CYP activities or mRNA's have been reported in the nervous system but these are predominantly of types metabolizing fatty acids and xenobiotics (subclasses CYP2C, 2D, 2E and 4). However, primary rat brain-derived glial cells have the capacity to synthesize pregnenolone and progesterone *in vitro*. Mellon and Deschepper, Brain Res. (1993), 629, 283-292(9)
20 provided molecular evidence for the presence, in brain, of key steroidogenic enzymes CYP11A1 (scc) and CYP11B1 (11 β) but failed to detect CYP17 (c17) or CYP11B2 (AS). Although CYP21A1 (c21) activity is reported to be present in brain, authentic CYP21A1 transcripts were not detected in this tissue.

Interest in steroid metabolism in brain has been fuelled by the finding that adrenal-
25 and brain-derived steroids (neurosteroids) can modulate cognitive function and synaptic plasticity. For instance, pregnenolone and steroids derived from it are reported to have memory enhancing effects in mice. However, the full spectrum of steroid metabolizing CYP's in brain and the biological roles of their metabolites *in vivo* has not been established.

Many aspects of brain function are modulated by steroids. Intracellular receptors
30 for glucocorticoids (cortisol, corticosterone) are particularly abundantly expressed in the

hippocampus (1), a brain region that plays a key role in specific aspects of memory formation, and which is an early and prominent target for dysfunction and damage in Alzheimer's disease (AD). While glucocorticoids regulate learning and memory, mood and neuroendocrine control, chronic glucocorticoid excess compromises neuronal activity, synaptic plasticity and eventually survival, particularly in the hippocampus. These findings prompted the suggestion that glucocorticoid-mediated neurotoxicity might underpin some age-related brain disorders, including AD, in which plasma cortisol levels are markedly elevated (2).

Conversely, dehydroepiandrosterone (DHEA), the most abundant steroid product of the human adrenal cortex, has been proposed to protect against disorders of the aging brain (3). Plasma levels of DHEA often show a striking age-associated decline which correlates with loss of cognitive function (4). In rodents, injection of DHEA or its sulfate into limbic structures improves post-training memory and enhances synaptic plasticity (5). DHEA and glucocorticoids thereby appear to exert inverse effects upon memory function and synaptic plasticity, and DHEA has been advocated as an endogenous 'anti-glucocorticoid'. However, despite considerable circumstantial evidence to support this contention, there is no evidence for a direct interaction between DHEA and glucocorticoid signalling pathways in neurons.

Neurosteroidogenesis has been reported in isolated rat retina (8) and brain (9). In addition to the production of pregnenolone and DHEA from cholesterol, a variety of novel steroids are made in brain extracts or cultured brain cells, including 20 α -dehydropregnenolone, 7 α -hydroxy derivatives of pregnenolone and DHEA, progesterone, and both 3 α - and 3 β -hydroxy-5 α -pregnan-20-one (reviewed in Ref. 7). Androgens are also modified, particularly through the action of aromatase and a 5 α -reductase (reviewed in Ref. 10). However, the specific enzymes responsible for these and other transformations in the central nervous system have not been well characterized.

As referred to above, several Cyps are present in the central nervous system (11-22). Activities or mRNAs corresponding to key steroidogenic enzymes (23-25), in addition to Cyp19 (aromatase) have been detected. Furthermore, mRNAs encoding the non-Cyp hydroxysteroid dehydrogenases (HSD) 3 α -HSD, 3 β -HSD and 11 β -HSD have been reported in the central nervous system (25, 27-29).

To investigate regulation of brain function, studies reported in copending International Patent Application No PCT/GB95/02465, published as WO 96/12810, and in Stapleton *et al* (J. Biol. Chem. 270, 29739 - 1995, December, 15 1995), focused on the hippocampus, a brain region important in learning and memory. A copy of the specification
5 of International Patent Application No PCT/GB95/02465 has been filed with the priority documents filed in respect of this specification.

That copending application, PCT/GB95/02465, describes and claims novel cytochrome P450 proteins designated Hct-1. These Hct-1 proteins have now been named as Cyp7b by the Committee on Standardized Cytochrome P450 Nomenclature and the
10 name Cyp7b will be used in this application.

The Cyp7b enzyme shares 39% sequence identity to hepatic cholesterol 7 α -hydroxylase (Cyp7a) and lesser but significant homology with other steroidogenic Cyps. The postulated steroidogenic domain (30,31), found in many of these enzymes, is present in both Cyp7a and Cyp7b. Cyp7b mRNA is predominantly expressed in rodent brain,
15 particularly in the hippocampus, unlike Cyp7a, which is liver-specific (31-33 and EP0648840 A2).

The present inventors have now investigated the substrate specificity of Cyp7b and found that Cyp7b catalyses the introduction of a hydroxyl group at the 7 α position in steroid substrates, particularly 3 β -hydroxy steroids. Cytochromes Cyp7b are thus steroid
20 hydroxylase enzymes having 7 α -specificity. The ability to produce 7 α -hydroxylated steroids is of major commercial importance, because such steroids are of particular use in the manufacture of pharmaceuticals (either as drugs *per se* or as intermediates), and in the manufacture of test kits and assays for pathological conditions associated with the presence of abnormal levels of endogenous enzyme, substrate or product.

25 The abbreviation "DHEA" will be used herein to designate dehydroepiandrosterone, thus 7 α -hydroxy-DHEA designates 7 α -hydroxydehydroepi-androsterone

The present inventors have identified substrate/product pairs associated with Cyp7b, particularly DHEA/7 α -hydroxy-DHEA (7-HD), pregnenolone/7 α -hydroxy-pregnenolone (7-HP) and β -estradiol/7 α -hydroxy- β -estradiol (7-HE). They have also determined that
30 DHEA concentration in brain tissue declines with age, whereas the concentrations of other brain steroids do not, and determined that the ageing process may be associated with

deficits in certain steroids and also with deficits in the concentration of Cyp7b itself. It is also believed that one of the products produced by Cyp7b mediated reactions, namely 7 α -hydroxy dehydroepiandrosterone, plays an important role in the operation of the immune system. Because 7 α -hydroxy-DHEA is believed to be made substantially only in the brain, the inventors hypothesize that senescence may be due to a deficit in
5 the brain-produced 7 α -hydroxy-DHEA as well as in other steroids found in the brain such as DHEA, pregnenolone and 7 α -hydroxy-pregnenolone.

The present inventors have now further determined that one of the specific properties of the 7 α -hydroxy-substituted steroids, and potentially their 7-oxo substituted
10 steroid derivatives, provided by the present invention is that of glucocorticoid and/or mineralocorticoid antagonism, whether at receptor level or otherwise. This is particularly demonstrated by the Example 5 below with respect to 7 α -hydroxy-DHEA but is more generally applicable. Thus this activity not only gives further uses for the novel steroids of the invention but provides first and second medical uses for known 7 α -hydroxy or 7-oxo
15 steroids made available by the present process as glucocorticoid and/or mineralocorticoid antagonists and preferably in antagonism specific to neuronal tissue such as in the CNS.

Thus, having regard to this activity and their involvement in endogenous metabolic pathways, particularly in the brain, the 7 α -hydroxy substituted 3 β -hydroxy-steroids provided by use of the Cyp7b enzyme activity, including novel compounds provided by the
20 invention, and their 7-oxo derivatives, have utility in the therapy of neuropsychiatric, immune and endocrine disorders, particularly but not exclusively steroid associated disorders.

Use of these 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroids, preferably possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or
25 derivatives thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in treating these disorders and for manufacturing medicaments for such treatment is provided in a first aspect of the present invention. Particularly preferred derivatives are those wherein one or both of the ester and or ether group is metabolisable *in vivo* to produce the corresponding hydroxy compound.

30 Preferred derivatives include those wherein the steroid has a 3 β -substituent-OR₁ and/or a 7 α -substituent -OR₂ where -OR₁ and -OR₂ each independently represents a free

hydroxy, ester or ether group,

wherein each of R_1 and R_2 are independently selected from the group consisting of hydrogen, substituted or unsubstituted C_{1-6} alkyl groups, groups R_5CO- , wherein R_5 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, and groups of the formula $-OP(OH)_3$, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) amino, C_{1-6} alkylamino, C_{1-6} dialkylamino, COOH or $COOR_4$ wherein R_4 represents a C_{1-6} alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

The particular disorders for which this utility is provided include

- 10 (a) deficits of cognition in aging
- (b) Alzheimer's disease
- (c) deficits of immune system in aging
- (d) deficits of immune function in HIV infection
- (e) glucocorticoid or mineralocorticoid excess
- 15 (f) diabetes
- (g) depression
- (h) osteoporosis and hypercalcemia
- (i) hyperglycemia and hyperlipodemia
- (j) muscle atrophy
- 20 (k) arterosclerosis
- (l) steroid diabetes

Further, these 7α -hydroxy steroids, their esters, ethers and 7-oxo derivatives may be used to induce cognitive enhancement in a normal individual.

Preferred steroids for such use have the carbon skeleton of androsterone, pregnenolone or estradiol and particularly preferred examples are 7α -hydroxy-DHEA and 7α -hydroxypregnenolone. Accordingly the present invention further provides the use of novel compounds of Formula Ia and Ib shown below in the applications indicated above.

Particularly preferred uses for the antagonistic properties of these 7-substituted steroids include treatment of disorders falling within category (e) above or where reversal of the effects of such corticoids, regardless of excess, is required.

A second aspect of the present invention provides pharmaceutical compositions implementing such use. The compositions in which the novel steroids and known steroids of the invention will be used will readily occur to those skilled in the art, generally comprising the steroid active in association with a pharmaceutically acceptable carrier or diluent, with formulations for example being suitable for inhalation or for gastrointestinal (eg. oral), parenteral, topical, transdermal or transmucosal administration.

As an alternative to administering the compounds of the invention *per se*, a third aspect of the invention provides the possibility of using the gene sequences of the Cyp7b genes in gene therapy in order to compensate for a deficiency in Cyp7b enzyme. In such therapies, constructs comprising Cyp7b coding sequences can be packaged in conventional delivery systems, such as adenoviruses, vaccinia viruses, herpes viruses and liposomes and administered via a route which results in preferential targeting of a selected tissue, especially the brain. The invention further provides the possibility of using the gene sequences of the Cyp7b genes in gene therapy in order to achieve the endogenous expression of Cyp7b sequences for other purposes, e.g. in order to promote immunogenic processes. Thus for example, a vector such as a suitably modified vaccinia virus (or variant thereof) may be co-administered with a vaccine formulation so that the expressed Cyp7b sequences augment the immunogenic properties of the vaccine.

It will be realised that in the event of Cyp7b related disorders other than those involving its depletion it may be desirable to use vectors containing antisense sequences to Cyp7b effective such as to inhibit Cyp7b expression.

Macromolecules related immunologically to Cyp7b enzymes form fourth and fifth aspects of the invention and in this regard antibodies, particularly monoclonal antibodies which are capable of selectively binding Cyp7b, have utility in the diagnosis of disorders (a) to (l) referred to above. Anti-Cyp7b antibodies (including monoclonal antibodies) as well as binding molecules comprising antibody fragments may be produced by known methods and used in test kits for assays for Cyp7b enzymes.

According to a sixth aspect of the invention, there is provided a process of producing a 7α -hydroxy-substituted steroid which comprises subjecting a corresponding steroid substrate having no hydroxyl substituent in the 7-position to hydroxylation in the presence of a Cyp7b steroid hydroxylase enzyme.

The Cyp7b steroid hydroxylase enzyme used in the process of the invention is preferably a Cyp7b enzyme described and claimed in the above-mentioned International Patent Application No PCT/GB95/02465 (and referred to therein as Hct-1). Such enzymes include (a) ones having the precise amino acid sequences described for mouse, rat and human Cyp7b, (b) homologous enzymes from other species and (c) enzymes having amino acid sequences which differ from the sequences of enzymes included in definitions (a) and (b), but in which the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.

The amino acid sequence of suitable Cyp7b steroid hydroxylase enzymes may be defined in terms of the DNA coding sequences disclosed in International Patent Application No PCT/GB95/02465. Thus the Cyp7b steroid hydroxylase enzyme may have a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from

- (a) Coding sequences of DNA molecules comprising the coding sequence for rat Cyp7b set forth in SEQ Id No: 1,
- 15 (b) Coding sequences of DNA molecules comprising the coding sequence for mouse Cyp7b set forth in SEQ Id No: 2,
- (c) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a) or (b) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 20 (d) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a), (b) or (c) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

The sequences (a) and (b) above represent rat and mouse Hct-1 gene sequence. Homologous sequences from other vertebrate species, especially mammalian species (including man) fall within the class of DNA molecules represented by (c) or (d).

Thus for human Cyp7b, the steroid hydroxylase enzyme may comprise a sequence encoded by

- (e) DNA coding sequences selected from the following:
- (i) the sequence designated "exon 3" in SEQ Id No 3,
 - (ii) the sequence designated "exon 4" in SEQ Id No 3, and
- (f) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (e) under standard hybridization conditions defined as 2 x SSC at 65°C.
- (g) Cyp7b steroid hydroxylase encoding DNA molecules capable of hybridizing with the DNA molecule defined in (e) or (f) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.
- (h) Cyp7b steroid hydroxylase-encoding DNA molecules comprising contiguous pairs of sequences selected from
- (i) the sequence designated "exon 3" in SEQ Id No 3,
 - (ii) the sequence designated "exon 4" in SEQ Id No 3, and
- (i) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (h) under standard hybridization conditions defined as 2 x SSC at 65°C.
- (j) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (h) or (i) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

- (k) Coding sequences of DNA molecules comprising a contiguous coding sequence consisting of the sequences "exon 3" and "exon 4" in SEQ Id No 3, and
- 5 (l) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (k) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 10 (m) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (k) or (l) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.

It will be appreciated that the DNA sequences referred to may consist of or be derived from genomic DNA, but typically would consist of or be derived from cDNA. Such sequences could be obtained by probing an appropriate library (cDNA or genomic) using hybridisation probes based upon the sequences provided according to the invention of International patent application No PCT/GB95/02465, or they could be prepared by chemical synthesis or by ligation of sub-sequences.

15 In the above definitions, Cyp7b steroid hydroxylases have been defined in terms of DNA sequence information. The Cyp7b steroid hydroxylase enzyme used in accordance with the process of the invention may alternatively or additionally be defined by reference to amino acid sequence information, e.g. the amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO 6.

20 Thus the Cyp7b steroid hydroxylase enzyme used in accordance with the process of the invention may have sequences matching one of said sequences exactly, or alternatively, the enzymes used may have sequences which differ from the aforementioned sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.

25 Thus, for example, mutant enzymes may be produced by known methods, for example site-directed mutagenesis or other PCR-based procedures, and the expression

products tested for their capacity to catalyse the introduction of a 7 α -hydroxyl group in selected substrates in accordance with the procedures described herein.

Having regard to the degree of homology between the rat, mouse and human enzymes and known data relating to species divergence of hydroxylase enzymes, it is preferred that by comparison with the DNA sequences of SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO.3, the mutant enzymes should be encoded by sequences having at least 50% homology, more preferably at least 60% homology and most preferably at least 70% homology with said sequences over a length of 50 contiguous nucleotides.

Preferably the mutant enzymes are encoded by sequences having at least 60% homology with the entire coding sequence, more preferably at least 70%.

Alternatively, by comparison with the amino acid sequences of SEQ ID NO. 4, SEQ ID NO. 5 and SEQ ID NO.6, it is preferred that mutant enzymes should have at least 50% homology, more preferably at least 60% homology and most preferably at least 70% homology with said sequences over a length of 30 contiguous amino acids. Preferably the mutant enzymes have at least 60% homology and more preferably 70% homology or more with the entire amino acid sequence in each case.

It is however preferred that such mutant enzymes do not differ too drastically from the aforementioned sequences and in this regard, where amino acid substitutions are made, that the substituted amino acids are preferably so-called "synonymous" or "conservative" substitutions, i.e. hydrophilic, hydrophobic, basic and acidic amino acids should preferably be substituted by amino acids in the same class (see US 5380712).

More specifically, it is preferred that the mutant enzymes differ from the precise sequences of those described herein by not more than 20, preferably not more than 10 and most preferably not more than 5 amino acid substitutions, insertions or deletions.

The Cyp7b enzymes described herein may be used in toxicological and drug evaluation studies and such uses form further aspects of the invention. In a particularly preferred embodiment of this aspect of the invention, a cell line capable of expressing a Cyp7b enzyme is used as a basis of an assay for one or more Cyp7b substrates. Such cell lines have utility in toxicological and drug evaluation studies. Most preferably the cell line comprises a prokaryotic or eucaryotic cell line which has been transformed so as artificially to express a Cyp7b enzyme. Examples include bacteria, yeast and mammalian cells. Also

included are transgenic animals, at least one tissue of which (especially a non-brain tissue) expresses Cyp7b enzyme. Such transgenic animals may be produced by known methods for introducing foreign coding sequences into somatic or germ line cells.

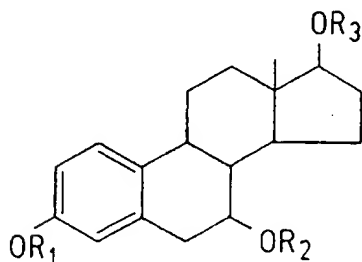
The substrates used in the method of the invention are characterised by possessing
 5 a 3β -hydroxyl group and further by preferably possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with the proviso that where the substrate has the carbon skeleton of cholesterol, the substrate has a hydroxyl group in the 25, 26 or 27-position, preferably the 25-position.

Examples of such substrates include 25-hydroxycholesterol, dehydroepi-
 10 androsterone, pregnenolone and estradiol, in which case the steroids produced will be 7α -hydroxy-25-hydroxycholesterol, 7α -hydroxydehydroepiandrosterone, 7α -hydroxy pregnenolone and 7α -hydroxyestradiol (i.e. estra 1,3,5(10)-triene-3,7 α ,17 β -triol) respectively.

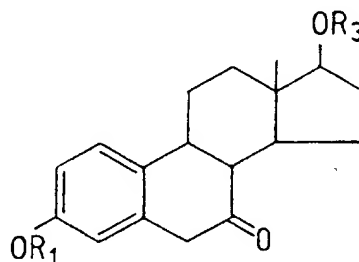
The 7α -hydroxylated steroid produced according to the invention may be oxidised
 15 by known enzymatic or non-enzymatic procedures to produce 7-oxo substituted steroids and this further process step forms a further aspect of the invention.

Certain 7α -hydroxy-substituted steroids produced according to the invention and certain corresponding 7-oxo derivatives are novel and provide a further aspect of the invention. Thus the present invention further provides novel 3β -hydroxy steroids
 20 characterised in that they have a 7α -hydroxy or 7-oxo substituent. Preferred novel steroids have the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with the proviso that where the skeleton is that of cholesterol, the 25, 26 or 27 position is hydroxylated, most preferably the 25 position.

Particular novel steroids are of the formula



Ia



Ib

wherein OR_1 , OR_2 and OR_3 each independently represents a free hydroxy group, an ether group or an esterified hydroxy group.

In the case where OR_1 , OR_2 and OR_3 each independently represents an ether group, each of R_1 , R_2 and R_3 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C_{1-6} alkylamino, C_{1-6} dialkylamino, COOH or $COOR_4$ wherein R_4 represents a C_{1-6} alkyl group which may be unsubstituted or substituted by one of the substituents referred to above.

In the case where OR_1 , OR_2 and OR_3 each independently represents an esterified hydroxy group, each of R_1 , R_2 and R_3 may have the formula R_5CO- , wherein R_5 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C_{1-6} alkylamino, C_{1-6} dialkylamino, COOH or $COOR_4$ wherein R_4 represents a C_{1-6} alkyl group; and groups of the formula $-OP(OH)_3$. Where compounds of Formula Ia or Ib include substituents such as carboxyl groups, phosphate groups, or substituted or unsubstituted amino groups, the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions (such as, for example, phosphate or halide ions) or cations (such as, for example, alkaline metal cations). Thus, where OR_1 , OR_2 or OR_3 represents hemesuccinate $HOOC(CH_2)_2CO$, the resulting hemesuccinate may be in the form of, for example, an Na or K salt.

It will be realised that the present invention provides for 7α -hydroxylated and 7-oxo steroids as described above but which are further substituted at other positions directly on the steroid skeleton.

7α -Hydroxyestradiol and 7-oxoestradiol are specific examples of compounds of Formula Ia and Ib.

The invention will now be described in more detail with particular reference to the following Figures and Examples.

Description of Figures

Figure 1 illustrates an autoradiogram of a TLC plate used in an experiment to assess the ability of various cell extracts to hydroxylate DHEA.

Figure 2 depicts the ability of various tissues to release radioactivity from 7-³H-pregnenolone.

Figure 3 illustrates the principal steroid interconversions mediated by Cyp7b.

Figure 4 is a histogram plotting fold induction of luciferase expression with
5 concentration of various steroids as described in Example 5.

Figure 5 illustrates the attenuation of Cyp7b gene expression in Alzheimer's as described in Example 5.

Figure 6 shows mass spectrometer plots of 7 α -hydroxy-DHEA produced by the present process and a reference sample thereof.

10 **EXAMPLE 1 - Identification of substrate specificity of Mu Cyp7b**

A. Preparation of vaccinia expression construct

To identify the reaction catalysed by Cyp7b a cDNA encoding the mouse enzyme, reported by Lathe, Rose and Stapleton (PCT/GB95/02465) and by Stapleton et al. (J. Biol. Chem. 270, 29739-1995, December 15 1995), was modified to introduce a translation
15 initiation consensus sequence at the 5' end of the Cyp7b open reading frame as described therein. The modified cDNA was introduced into the genome of vaccinia virus by recombinational exchange according to standard procedures (see, for instance, Gonzalez et al., Meth. Enzymol. 206, 85-92, 1991 and references therein) as described in Lathe et al.

20 **B. Production of Cyp7b enzyme extracts.**

Hela cells were grown to semi confluence (10⁶ cells per 5 cm dish; 5 ml medium) and infected with recombinant (VV-Cyp7b) and control (VV Copenhagen strain) vaccinia viruses at 0.1 pfu per cell; 16 hours later infected cells were washed and taken up into W (Waxman) buffer (0.1 M KP04, 1 mM EDTA, 20% glycerol pH 7.5; 500 μ l per plate) and
25 recentrifuged (5 min., 1000 rpm).

For whole cell extracts cells were resuspended into 1/100 volume (50 μ l per plate) of W buffer and stored frozen at -70°C. For microsome preparation (Waxman, Biochem. J. 260, 81-85, 1989) cells were resuspended in 1/10 original volume of W buffer (500 μ l per plate); sonicated 6 x 5 seconds on ice, and unbroken cells were removed by
30 centrifugation (10 min., 4°C, 3000 rpm).

The microsomal fraction was prepared from the supernatant by centrifugation

(100,000 g, 45 min., 4°C, Beckman SW50.1 rotor) and resuspended using a Potter homogeniser in 1/50 original volume of W buffer (100 µl per plate) before storage at -70°C.

Control extracts were prepared from liver and brain from male rat by homogenising
5 fresh tissue in W buffer (2.5 ml/g), clarifying briefly by centrifugation (4000 rpm, 5 min, 4°C); the supernatant was stored at -70°C.

C. Substrate identification by thin-layer chromatography.

¹⁴C or ³H-labelled steroids were purchased from DuPont-NEN (¹⁴C-labelled
10 molecules: specific activities 45-60 mCi/mmol.; ³H: specific activities 70-100 mCi/mmol).
1 nMol aliquots of labelled substrate were dried down, microsomes or cell and tissue
extracts were added (25 to 50 µl), and diluted to a volume of 175 µl with W buffer.

Reaction was started by the addition of 25 µl of 8 mM NADPH. After incubation
at 37°C for 15 minutes the reaction was shaken with 500 µl of ethyl acetate (BDH). The
15 organic phase was removed, dried down, and suspended into 10 µl ethyl acetate. Aliquots
(5 x 2 µl) were applied to thin layer chromatography (TLC) sheets (Merck) and developed
in ethyl acetate/n-hexane/acetic acid 16:8:1 (solvent system N of Waxman, Meth. Enzymol.
206, 462-476, 1991). After drying, chromatograms ¹⁴C were visualised by exposure to
X-ray film. ³H-labelled chromatograms were treated with EN³ HANCETM (DuPont-NEN)
20 spray prior to exposure.

D. Results

Figure 1 is an autoradiogram of a TLC plate run in solvent system N; substrate was
³H-DHEA and samples were extracted with ethyl acetate and dried prior to application to
the TLC plate (origin at bottom of figure). Extracts were 1, Microsomes from Hela cells
25 infected with control vaccinia virus (negative control); 2, Microsomes from Hela cells
infected with VVCyp7b; 3, Duplicate preparation of microsomes from Hela cells infected
with VVCyp7b; 4, Rat brain homogenate.

As can be seen from Figure 1, microsomes from cells infected with recombinant
vaccinia expressing Cyp7b converted ¹⁴C-dehydroepiandrosterone (DHEA) to a lower
30 mobility form most consistent with hydroxylation. Brain extracts yielded a product of
indistinguishable mobility, consistent with our earlier demonstration that Cyp7b is

expressed in brain. From the relative mobility of the product we surmised that Cyp7b could be hydroxylating DHEA at the 7 position. Progesterone, corticosterone, cortisol and testosterone were at best inefficiently metabolised, if at all. However, pregnenolone and estradiol were both converted by the enzymes, as was 25-hydroxy cholesterol. All these
5 substrates are distinguished by a 3 β hydroxy group.

EXAMPLE 2 - Identification of the position of the modification by ^3H -release.

To identify the position of the modification, ^3H -pregnenolone (NEN) was employed in which the ^3H substitution was predominantly at the 7 position on the steroid backbone. Microsomal extracts were incubated with ^3H -pregnenolone under the same conditions as
10 used earlier. Following reaction, labelled steroids were extracted with ethyl acetate (2 x 1 ml), and discarded; release of ^3H into the aqueous phase was monitored by liquid scintillation counting.

Referring to Figure 2, 7- ^3H -pregnenolone was incubated with extracts and assayed for release of radioactivity into the aqueous phase following extraction with ethyl acetate.
15 Extracts were 1, Microsomes from Hela cells infected with control vaccinia virus (negative control); 2, Microsomes from Hela cells infected with VVCyp7b; 3, Duplicate preparation of microsomes from Hela cells infected with VVCyp7b; 4, Rat brain homogenate; 5, Rat liver homogenate.

As seen in Figure 2 microsomes from cells infected with recombinant
20 vaccinia expressing Cyp7b efficiently released ^3H into the aqueous phase. Brain also performed this reaction but not liver. Release of ^3H from the 7 position of pregnenolone demonstrates that Cyp7b hydroxylates pregnenolone at the 7-position to generate 7-hydroxy pregnenolone (7HP); it may be concluded that Cyp7b also hydroxylates DHEA (to generate 7-hydroxy DHEA [7HD]) and estradiol to generate 7-hydroxy estradiol [7HE].

25

EXAMPLE 3 - Stereochemistry of the Cyp7b hydroxylation.

Steroids hydroxylated at a variety of positions (egs. 2, 6, 7, 15, 16) differ in their mobility on TLC depending on whether the modification is in the α - or the β -position (Waxman, Meth. Enzymol. 206, 462-476, 1991). Purified 7 α -hydroxy DHEA was
30 obtained (kind gift of Dr. H.A. Lardy, Enzyme Institute, University of Wisconsin), mixed with the product of Cyp7b action on DHEA, and subjected to TLC. The product

comigrated with 7 α -hydroxy-DHEA, demonstrating that Cyp7b is a 7 α hydroxylase.

EXAMPLE 4 - Activity of enzyme in 7 α -hydroxylation of pregnenolone and DHEA

To examine the catalytic activity of the enzyme Cyp7b CDNAs were expressed in mammalian cell lines. Cell extracts showed substantial NADPH-dependent conversion of DHEA (Km 13.3 μ M; Vmax 288pmol/min/mg) and pregnenolone (Km 3.6 μ M; Vmax 34 pmol/min/mg) to slower migrating forms on thin layer chromatography. Products of identical mobility were generated by rat brain extracts. The expressed enzyme was less active against 25-hydroxycholesterol, 17 β -estradiol and 5 α -androstane-3 β , 17 β -diol, with low to undetectable activity against progesterone, corticosterone and testosterone. When [3H-7 α] pregnenolone was incubated with Cyp7b extracts the extent of release of radioactivity into the medium suggested that hydroxylation was preferentially at the 7 α -position. In gas chromatography and mass spectrometry of the modified steroid arising from incubation of DHEA with Cyp7b extracts, the retention time and fragmentation patterns were identical to those obtained with authentic 7 α -hydroxy DHEA (7HD); the reaction product also co-migrating with 7HD on TLC.

Mass spectrometry: A 10x scaled up reaction was employed using 95% unlabelled DHEA (Sigma) and 5% [14C]-DHEA (final specific activity 2.25-3mCi/mmol) and reaction time was extended to 1 hour. Product was purified by TLC, excised and extracted with ethyl acetate before drying down. The dried residue and authentic 7HD (50mg) were converted to their methoxime -trimethylsilyl (MO-TMS) derivatives. Analysis of these products was performed using a Trio 100 mass spectrometer operating in electron impact (EI) mode, linked to a HP5890 gas chromatograph fitted with a HP-1 cross-linked methyl siloxane column (25m, i.d. 0.25mm, 0.17 mm film) under the following conditions: electron energy 70eV, source temperature 200°C, interface temperature 280°C, oven temperature 50°C increasing at 30°C per minute to 200°C, and then 10°C per minute to 300°C, injection temperature 280°C.

EXAMPLE 5 - Cis-trans co-transfection assay; demonstration of antagonism.

Chinese hamster ovary (CHO) cells were maintained and transfected in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 15% foetal bovine serum, 100IU/ml penicillin, 100 μ g/ml streptomycin and 200mM L-glutamine (all Gibco BRL,

Paisley, UK).

24 hours prior to transfection CHO cells were plated at a density of 3×10^5 /60 mm dish (Costar UK). Cells were transfected by the calcium phosphate method. Briefly, 5µg of MMTV-LUC and 1µg of pRShGR or 5µg of pSV2 as a control for transfection efficiency were made up to a total of 10µg/plate of DNA with pGEM3. 30µl of 2.5M CaCl_2 was diluted ten-fold with sterile water and 300µl was added to the DNA. Next 300µl of 2 x Hepes buffered saline (280 mM NaCl, 10mM KCl, 1.5mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), 50mM Hepes, 12mM dextrose, pH 7.05) was added slowly with swirling to the DNA/ CaCl_2 mixture. This solution was left for 30 minutes in order for a fine precipitate to form and 600µl was added dropwise to each plate. After 24 hours the medium was removed and the cells were washed in serum free medium and culture for a further 24 hours in medium containing 10% charcoal-stripped serum together with the appropriate concentrations of DHEA/7α-hydroxy-DHEA.

Six hours after the addition of DHEA/7α-hydroxyDHEA either B or Dex was added to each plate. The following day the cells were washed in PBS, lysed with 0.3ml of lysis buffer (25mM Tris-phosphate pH 7.8, 2mM DTT, 1% Triton X-100 and 10% glycerol), scraped, centrifuged and the supernatant assayed in duplicate in a Berthold luminometer in a total volume of 250µl, comprising 40µl of cell extract, 5µl of 30mM ATP, 100µl of assay buffer (20mM tricine, 1.07mM $(\text{MgCO}_3)_4 \cdot \text{Mg}(\text{OH})_2 \cdot \% \text{H}_2\text{O}$, 2.67mM MgSO_4 , 0.1mM EDTA, 33.3mM DTT, 0.2mg/ml coenzyme A) and 105µl luciferin (Promega UK) injected to initiate the reaction. Light emission was measured over 10 seconds and relative light units/microgram of protein was calculated.

Results are shown in Figure 4 wherein the fold induction of luciferase is illustrated by histogram for control, additions of DHEA, 7α-hydroxy-DHEA (7HD) alone and these additions in presence of an GR activating concentration of corticosterone. This result shows that 7HD, but not DHEA, acts as an antagonist of corticosterone effect in activating the GR-mediated transcription.

EXAMPLE 6 - Cyp7b expression in Alzheimers neurons

Cryostat brain sections (10µm) from control and Alzheimer's hippocampus were cut, thaw mounted onto gelatine-subbed poly-L-lysine coated slides and stored at -80°C.

For *in-situ* hybridization studies, brain sections were post-fixed in 4%

paraformaldehyde by acetylation (0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0) for 10 minutes, rinsed in phosphate buffered saline, dehydrated through graded alcohols and air dried. Hybridization was carried out using 200µl of [³⁵S]-UTP-labelled cRNA antisense probe (10 x 10⁶ dpm/ml in hybridization buffer) synthesized *in vitro* from a 500 bp XbaI/PstI fragment of the human Cyp7b pMMCTl clone linearised with XbaI and transcribed with T3 RNA for sense probes. Sections were prehybridized with 20µl of prehybridization buffer (as hybridization buffer but omitting the dextran sulphate) per slide at 50°C for 3 hours.

Following hybridization with probe at 50°C overnight sections were treated with RNase A (30µg/ml, 45 minutes at 37°C) and washed to a final stringency of 0.1 x SSC at 60°C. Slides were dehydrated, dipped in photographic emulsion (NTB-2, Kodak) and exposed at 4°C for 5 weeks before being developed and counterstained with 1% pyronin. The density of silver grains was assessed over individual hippocampal neurons by computer-assisted grain counting using an image analysis system (Seescan plc, Cambridge, UK), with the analysis carried out blind (sections were cut and coded by a separate individual). For each slide, one hippocampal section represents each subject. 6-10 neurons/subregion were assessed and background, counted over areas of white matter, was subtracted. Data were assessed by ANOVA followed by Scheffe post hoc test. Significance was set at p<0.05. Values are means ± S.E.M.

Figure 5 is a histogram showing Cyp7b expression as indicated by grain count per neuron in the dentate gyrus, CA1 and CA3 subfields of Alzheimer's disease samples as compared to the age matched control brains.

CONCLUSIONS

It can be concluded that Cyp7b, and cognate enzymes from rat, human and other mammalian species, are 7α-hydroxylases specific for steroid substrates with a 3β hydroxy group. While activities for 7-hydroxylating DHEA, pregnenolone and cholesterol have been recorded previously in a variety of crude tissue homogenates (eg. Akwa et al., Biochem. J. 288, 959-964, 1992) no characterisation of the enzyme responsible was performed previously and no activity on estradiol was recorded. Recombinant organisms expressing Cyp7b thus provide a route to the large scale manufacture of 7HP, 7HD, and 7HE, principally but not exclusively for therapeutic use or for the production of further steroid derivatives such as 7-oxo molecules.

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CLAIMS

1. The use of a 7α -hydroxy or 7-oxo substituted 3β -hydroxy-steroid, or a derivative thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in the manufacture of a pharmaceutical composition for the therapy of neuropsychiatric, immune and/or endocrine disorders or for inducing cognitive enhancement.
2. The use according to Claim 1 wherein said disorders are selected from
 - (a) deficits of cognition in aging
 - 10 (b) Alzheimer's disease
 - (c) deficits of immune system in aging
 - (d) deficits of immune function in HIV infection
 - (e) glucocorticoid or mineralocorticoid excess
 - (f) diabetes
 - 15 (g) depression
 - (h) osteoporosis and hypercalcemia
 - (I) hyperglycemia and hyperlipodemia
 - (j) muscle atrophy
 - (k) arterosclerosis
 - 20 (l) steroid diabetes
3. The use as claimed in claim 1 or claim 2 wherein the steroid has a 3β -substituent-OR₁ and/or a 7α -substituent -OR₂ where -OR₁ and -OR₂ each independently represents a free hydroxy, ester or ether group,
 - 25 wherein each of R₁ and R₂ are independently selected from the group consisting of hydrogen, substituted or unsubstituted C₁₋₆ alkyl groups, groups R₅CO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, and groups of the formula -OP(OH)₃, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a

C₁₋₆ alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

4. The use as claimed in any one of claims 1 to 3 characterised in that the steroid is one possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol,
5. The use of a Cyp7b steroid hydroxylase enzyme in the manufacture of a test kit for use in the diagnosis of neuropsychiatric, immune and endocrine disorders.
6. The use according to Claim 5 wherein said disorders are selected from
 - (a) deficits of cognition in aging
 - (b) Alzheimer's disease
 - 10 (c) deficits of immune system in aging
 - (d) deficits of immune function in HIV infection
 - (e) glucocorticoid or mineralocorticoid excess
 - (f) diabetes
 - (g) depression
 - 15 (h) osteoporosis and hypercalcemia
 - (I) hyperglycemia and hyperlipodemia
 - (j) muscle atrophy
 - (k) arterosclerosis
 - (l) steroid diabetes
- 20 7. An antibody, especially a monoclonal antibody, characterised by specifically binding Cyp7b enzymes.
8. The use of an antibody as claimed in Claim 5 in a test kit for assaying for the presence of Cyp7b enzymes.
9. The use of Cyp7b coding sequences or antisense sequences in the manufacture of
25 a targeted drug for gene therapy of Cyp deficiencies or excesses or for promoting immunogenic processes.

10. The use claimed in Claim 9 wherein a vector is co-administered with a vaccine formulation, whereby on administration, a Cyp7b sequence is expressed and the produced expression product augments an immunogenic property of the vaccine.
11. A process of producing a 7 α -hydroxy-substituted steroid which comprises
5 subjecting a corresponding steroid substrate having no substituent in the 7-position to hydroxylation in the presence of a Cyp7b steroid hydroxylase enzyme.
12. A process according to Claim 11 wherein the enzyme is a mouse, rat or human Cyp7b steroid hydroxylase enzyme.
13. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme
10 has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from
- (a) Coding sequences of DNA molecules comprising the coding sequence for rat Cyp7b set forth in SEQ Id No: 1,
 - (b) Coding sequences of DNA molecules comprising the coding sequence for mouse Cyp7b set forth in SEQ Id No: 2,
 - 15 (c) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (a) or (b) under standard hybridization conditions defined as 2 x SSC at 65°C.
 - (d) Cyp7b steroid hydroxylase-encoding DNA molecules capable of
20 hybridizing with the DNA molecule defined in (a), (b) or (c) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.
14. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from

- (e) DNA coding sequences selected from the following:
- (i) the sequence designated "exon 3" in SEQ Id No 3.
 - (ii) the sequence designated "exon 4" in SEQ Id No 3. and
- 5 (f) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (e) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 10 (g) Cyp7b steroid hydroxylase encoding DNA molecules capable of hybridizing with the DNA molecule defined in (e) or (f) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.
- (h) Cyp7b steroid hydroxylase-encoding DNA molecules comprising contiguous pairs of sequences selected from
- (i) the sequence designated "exon 3" in SEQ Id No 3,
 - (ii) the sequence designated "exon 4" in SEQ Id No 3, and
- 15 (i) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (h) under standard hybridization conditions defined as 2 x SSC at 65°C.
- 20 (j) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (h) or (i) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.
- (k) Coding sequences of DNA molecules comprising a contiguous coding sequence consisting of the sequences "exon 3" and "exon 4" in SEQ Id No 3, and

- (l) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecules defined in (k) under standard hybridization conditions defined as 2 x SSC at 65°C.
- (m) Cyp7b steroid hydroxylase-encoding DNA molecules capable of hybridizing with the DNA molecule defined in (k) or (l) under reduced stringency hybridization conditions defined as 6 x SSC at 55°C.
15. A process according to Claim 11 wherein the Cyp7b steroid hydroxylase enzyme has a sequence encoded by DNA coding sequences of Cyp7b enzymes selected from the amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO 6 or a sequence which has at least 50% homology with one or more of the aforementioned sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.
16. A process according to Claim 15 wherein the Cyp7b steroid hydroxylase enzyme has a sequence encoded by a DNA coding sequence which has at least 60% homology, and preferably at least 70% homology with one or more of the aforementioned sequences, provided that the capacity to catalyse the introduction of a 7 α -hydroxyl group is not eliminated.
17. A process according to Claim 15 wherein the Cyp7b steroid hydroxylase enzyme has a sequence which differs from the amino acid sequences contained in SEQ ID NO. 4, SEQ ID NO. 5 or SEQ ID NO 6 by not more than 20, preferably not more than 10 and most preferably not more than 5 amino acid substitutions, insertions or deletions.
18. A process according to any preceding claim wherein substrate is a steroid possessing a 3 β -hydroxyl group.
19. A process according to any preceding claim wherein the substrate is a steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, with

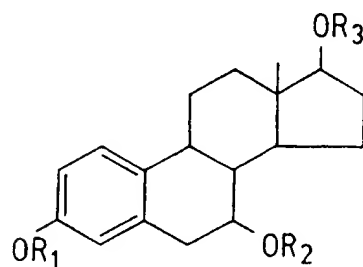
the proviso that where the substrate has the carbon skeleton of cholesterol, the substrate has a hydroxyl group in the 25, 26 or 27-position.

20. A process according to Claim 19 wherein the substrate is 25-hydroxycholesterol, dehydroepiandrosterone, pregnenolone or estradiol.

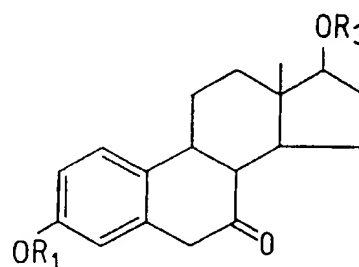
21. A process according to any preceding claim wherein the produced 7α -hydroxy-substituted steroid is 7α -hydroxyestradiol, 7α -hydroxypregnenolone or 7α -hydroxydehydroepiandrosterone.

22. A process according to any preceding claim wherein produced steroid is subjected to an oxidation step to convert an H.OH to an oxo group.

23. A steroid of the formula



Ia



Ib

wherein OR_1 , OR_2 and OR_3 each independently represents a free hydroxy group, an ether group or an esterified hydroxy group.

24. A steroid according to Claim 23 wherein

each of R_1 , R_2 and R_3 may be selected from substituted or unsubstituted C_{1-6} alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, (C_{1-6}) alkylamino, C_{1-6} dialkylamino, COOH or COOR₄ wherein R_4 represents a C_{1-6} alkyl group which may be unsubstituted or substituted by one of the substituents referred to above; or

OR₁, OR₂ and OR₃ each independently represents an esterified hydroxy group, of the formula R₅COO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, any such substituents being selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a C₁₋₆ alkyl group;

or

OR₁, OR₂ and OR₃ each independently represents an esterified hydroxy group of formula -OP(OH)₃,
or a pharmacologically acceptable salt of such a compound.

25. 7 α -Hydroxyestradiol or 7-oxoestradiol.

26. A steroid as claimed in Claim 23 characterised in that it is a 3 β -hydroxy steroid.

27. A process of producing an oxo-substituted steroid which comprises subjecting 7 α -hydroxyestradiol, 7 α -hydroxypregnenolone or 7 α -hydroxydehydroepiandrosterone to oxidation.

28. A method for treating a human or animal requiring therapy for a neuropsychiatric, immune and endocrine disorder or for inducing cognitive enhancement comprising the administration of an effective amount of a 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroid or derivative thereof independently substituted at one or both of the 7-and 3-positions by an ester or ether group.

20

29. A method according to Claim 28 wherein said disorders are selected from

- (a) deficits of cognition in aging
- (b) Alzheimer's disease
- (c) deficits of immune system in aging
- (d) deficits of immune function in HIV infection
- (e) glucocorticoid or mineralocorticoid excess
- (f) diabetes
- (g) depression

25

- (h) osteoporosis and hypercalcemia
- (I) hyperglycemia and hyperlipidemia
- (j) muscle atrophy
- (k) arterosclerosis
- 5 (l) steroid diabetes

30. A method as claimed in claim 28 wherein the steroid possesses the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol and has a 3β -substituent-OR₁ and/or a 7α -substituent -OR₂ where -OR₁ and -OR₂ each independently represents a free hydroxy, ester or ether group,

wherein each of R₁ and R₂ are independently selected from the group consisting of hydrogen, substituted or unsubstituted C₁₋₆ alkyl groups, groups R₅CO-, wherein R₅ may be selected from substituted or unsubstituted C₁₋₆ alkyl groups, and groups of the formula -OP(OH)₃, wherein any substituents are selected from OH, halogen (F, Cl, Br, I) amino, C₁₋₆ alkylamino, C₁₋₆ dialkylamino, COOH or COOR₄ wherein R₄ represents a C₁₋₆ alkyl group; and wherein the compounds may be in free form or in the form of acid addition salts with pharmacologically acceptable anions.

31. A 7α -hydroxy or 7-oxo substituted 3β -hydroxy-steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or a derivative thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group for use in therapy.

32. A steroid as claimed in claim 31 selected from 7α -hydroxydehydroepiandrosterone, 7α -hydroxypregnenolone and 7α -hydroxyestradiol.

33. A pharmaceutical composition characterised in that it comprises a 7α -hydroxy or 7-oxo substituted 3β -hydroxy steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or a derivative thereof substituted independently at one or both of the 7- and 3- positions with an ester or ether group, in association with a pharmaceutically acceptable carrier or diluent in a sterile and pyrogen free form.

SEQUENCE LISTING

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40 (B) STREET: Molecular Medicine Centre, The University of
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(C) CITY: Edinburgh
(E) COUNTRY: GB
(F) POSTAL CODE (ZIP): EH4 2XU

45 (ii) TITLE OF INVENTION: NEUROSTEROIDS

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

- 5 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: *****

10 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 1763 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix). FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1245

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

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25 GAG AAG TTC AGC CGA AGA TTA TCA GCG AAA GCC TTC TCT GTC AAG AAG
96
Glu Lys Phe Ser Arg Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys
    20             25             30

30 CTG CTA ACT AAT GAC GAC CTT AGC AAT GAC ATT CAC AGA GGC TAT CTT
Leu Leu Thr Asn Asp Asp Leu Ser Asn Asp Ile His Arg Gly Tyr Leu
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CTT TTA CAA GGC AAA TCT CTG GAT GGT CTT CTG GAA ACC ATG ATC CAA
Leu Leu Gln Gly Lys Ser Leu Asp Gly Leu Leu Glu Thr Met Ile Gln
    50             55             60

35 GAA GTA AAA GAA ATA TTT GAG TCC AGA CTG CTA AAA CTC ACA GAT TGG
Glu Val Lys Glu Ile Phe Glu Ser Arg Leu Leu Lys Leu Thr Asp Trp
    65             70             75             80

AAT ACA GCA AGA GTA TTT GAT TTC TGT AGT TCA CTG GTA TTT GAA ATC
Asn Thr Ala Arg Val Phe Asp Phe Cys Ser Ser Leu Val Phe Glu Ile
40             85             90             95

ACA TTT ACA ACT ATA TAT GGA AAA ATT CTT GCT GCT AAC AAA AAA CAA
Thr Phe Thr Thr Ile Tyr Gly Lys Ile Leu Ala Ala Asn Lys Lys Gln
    336

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5	CCA TAC TTA GTA TCT GAC ATA CCT ATT CAG CTT CTA AGA AAT GCA GAA			432
	Pro Tyr Leu Val Ser Asp Ile Pro Ile Gln Leu Leu Arg Asn Ala Glu			
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	TTT ATG CAG AAG AAA ATT ATA AAA TGT CTC ACA CCA GAA AAA GTA GCT			480
	Phe Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Pro Glu Lys Val Ala			
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15	Lys Lys Tyr Tyr Gly His Glu Glu Phe Glu Ile Gly Ala His His Leu			
	180	185	190	
	GGC TTG CTC TGG GCC TCT CTA GCA AAC ACC ATT CCA GCT ATG TTC TGG			624
	Gly Leu Leu Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala Met Phe Trp			
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20	GCA ATG TAT TAT CTT CTT CAG CAT CCA GAA GCT ATG GAA GTC CTG CGT			672
	Ala Met Tyr Tyr Leu Leu Gln His Pro Glu Ala Met Glu Val Leu Arg			
	210	215	220	
	GAC GAA ATT GAC AGC TTC CTG CAG TCA ACA GGT CAA AAG AAA GGA CCT			720
	Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro			
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	260	265	270	
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	Ile His Asn Asp Pro Glu Val Phe Asp Ala Pro Lys Asp Phe Arg Phe			
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	GAT CGC TTC GTA GAA GAT GGT AAG AAG AAA ACA ACG TTT TTC AAA GGA			1008
	Asp Arg Phe Val Glu Asp Gly Lys Lys Lys Thr Thr Phe Phe Lys Gly			

	325	330	335	
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5	AAA TGT CCA GGC AGA TAC TTT GCA ATT AAT GAA ATG AAG CTA CTA GTG			1104
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	ATT ATA CTT TTA ACT TAT TTT GAT TTA GAA GTC ATT GAC ACT AAG CCT			1152
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	Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp			
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	GCGATCCATC CAGTTTGTTT TGGGTCACAA AACCTGTCAT AAAATAAAGC GCTGTCATGG			1425
20	TGTAAAAAAA TGTCATGGCA ATCATTTCAG GATAAGGTAA AATAACGTTT TCAAGTTTGT			1485
	ACTTACTATG ATTTTATCA TTTGTAGTGA ATGTGCTTTT CCAGTAATAA ATTTGCGCCA			1545
	GGGTGATTTT TTTTAATTAC TGAAATCCTC TAATATCGGT TTTATGTGCT GCCAGAAAAG			1605
	TGTGCCATCA ATGGACAGTA TAACAATTTC CAGTTTTCCA GAGAAGGGAG AAATTAAGCC			1665
	CCATGAGTTA CGCTGTATAA AATTGTTCTC TTCAACTATA ATATCAATAA TGTCTATATC			1725
25	ACCAGGTTAC CTTTGCATTA AATCGAGTTT TGCAAAAG			1763

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1880 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 81..1604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGCAGGCACA GCCTCTGGTC TAAGAAGAGA GGGCACTGTG CAGAAGCCAT CGCTCCCTAC
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420 425

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Ser Pro Gly Pro Leu Ala Leu Leu Gly Leu Leu Phe Ala Ala Thr Leu
430 435 440

CTG CTC TCG GCC CTG TTC CTC CTC ACC CGG CGC ACC AGG CGC CCT CGT 206
10 Leu Leu Ser Ala Leu Phe Leu Leu Thr Arg Arg Thr Arg Arg Pro Arg
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GAA CCA CCC TTG ATA AAA GGT TGG CTT CCT TAT CTT GGC ATG GCC CTG 254
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15 AAA TTC TTT AAG GAT CCG TTA ACT TTC TTG AAA ACT CTT CAA AGG CAA 302
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475 480 485

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Val Lys Lys Leu Leu Thr Asp Asp Asp Leu Asn Glu Asp Val His Arg
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5	Asp Met Phe Pro Tyr Leu Val Ser Asp Ile Pro Ile Gln Leu Leu Arg	
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	AAT GAA GAA TCT ATG CAG AAG AAA ATT ATA AAA TGC CTC ACA TCA GAA	830
	Asn Glu Glu Ser Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Ser Glu	
	650 655 660 665	
10	AAA GTA GCT CAG ATG CAA GGA CAG TCA AAA ATT GTT CAG GAA AGC CAA	878
	Lys Val Ala Gln Met Gln Gly Gln Ser Lys Ile Val Gln Glu Ser Gln	
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	Asp Leu Leu Lys Arg Tyr Tyr Arg His Asp Asp Ser Glu Ile Gly Ala	
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	His His Leu Gly Phe Leu Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala	
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25	AAA GGG CCT GGA ATT TCA GTC CAC TTC ACC AGA GAA CAA TTG GAC AGC	1118
	Lys Gly Pro Gly Ile Ser Val His Phe Thr Arg Glu Gln Leu Asp Ser	
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	765 770 775	
30	TAC TCC AGC ATC ATC CGA GAA GTG CAG GAG GAT ATG AAT CTC AGC TTA	1214
	Tyr Ser Ser Ile Ile Arg Glu Val Gln Glu Asp Met Asn Leu Ser Leu	
	780 785 790	
	GAG AGT AAG AGT TTC TCT CTG CGG AAA GGA GAT TTT GTA GCC CTC TTT	1262
35	Glu Ser Lys Ser Phe Ser Leu Arg Lys Gly Asp Phe Val Ala Leu Phe	
	795 800 805	
	CCT CCA CTC ATA CAC AAT GAC CCG GAA ATC TTC GAT GCT CCA AAG GAA	1310
	Pro Pro Leu Ile His Asn Asp Pro Glu Ile Phe Asp Ala Pro Lys Glu	
	810 815 820 825	
40	TTT AGG TTC GAT CGG TTC ATA GAA GAT GGT AAG AAG AAA AGC ACG TTT	1358
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	830 835 840	
	TTC AAA GGA GGG AAG AGG CTG AAG ACT TAC GTT ATG CCT TTT GGA CTC	1406
	Phe Lys Gly Gly Lys Arg Leu Lys Thr Tyr Val Met Pro Phe Gly Leu	

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	860	865	870	
5	CTA CTG CTG ATT GAG CTT TTA ACT TAT TTT GAT TTA GAA ATT ATC GAC			1502
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	Arg Lys Pro Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln			
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	CAC CCC GAT TCT GCC GTC TCC TTT AGG TAC AAA GCA AAA TCT TGG AGA			1598
	His Pro Asp Ser Ala Val Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg			
	910	915	920	
	AGC TGA AAGTGTGGCA GAGAAGCTTT GCAGAGTAAG GCTGCATGTG CTGAGCTCCG			1654
15	Ser *			
	TGATTTGGTG CACTCCCCCA AATGCAACCG CTACTCTTGT TTGAAAATGG CAAATTTATA			1714
	TTTGGTTGAG ATCAATCCAG TTGGTTTTGG GTCACAAAAC CTGTCATAAA ATAAAGCAGT			1774
	GTGATGGTTT AAAAAATGTC ATGGCAATCA TTTCAGGATA AGGTAAAATA ACATTTTCAA			1834
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3846 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:831..2078

(ix) FEATURE:

(A) NAME/KEY: exon (3)

(B) LOCATION:831..1422

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:1423..1872

(ix) FEATURE:

(A) NAME/KEY: exon (4)

(B) LOCATION:1873..2078

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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TTTGGCCAGT GTTGAATTAG ACATTATTT GTGAGTACCT GCTCCATACA GTATGGTCAT 240

10 TTATTTGAGT TAAATTTGTT GTATTTGAAC AAAACTCAGA TGACACCTAA GCATGAAAAA 300

GCTCTTTATG AAGTATAAAT ACTCAGAAAT GGAATGGCAT GTTGCCAATT TGTTTCTGC 360

TTTATTGAGG GAAATATATG AGAAGTATTT AAGTCAGGGG ATTATGAGGA ATATTTAAAG 420

GATANNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 480

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 540

15 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 600

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNTCTAGA GTGTTTCCA CCATCTTTCA 660

AAGGAACAT GTAGTGACC TTCGAATGAA ATGGATTTGT ATTAACTTT TTGCCTTAGT 720

TATTAGGGTC TTTCTAATTT TTGATTAACA TATTTTTTTA ATTTGTGGTG TTTATTTCTG 780

TTTTTATTAA CAAACGAAC CATATGCTCC TCTCTCTTT TTTTCTTCT GGAAAGTACA 840

20 TAACATTTAT ACCTGGACCC TTCCAGTACC AGCTAGTGAT AAAAAATCAT AAACAATTAA 900

GCTTTCGAGT ATCTTCTAAT AAATTATCAG AGAAAGCATT TAGCATCAGT CAGTTGCAAA 960

AAAATCATGA CATGAATGAT GAGCTTCACC TCTGCTATCA ATTTTGTCAA GGCAAATCTT 1020

TGGACATACT CTTGGAAAGC ATGATGCAGA ATCTAAAACA AGTTTTTGAA CCCCAGCTGT 1080

TAAAAACCAC AAGTTGGGAC ACGGCAGAAC TGTATCCATT CTGCAGCTCA ATAATATTTG 1140

25 AGATCACATT TACAACTATA TATGAAAAAG TTATTGTTTG TGACAACAAC AAATTTATTA 1200

GTGAGCTAAG AGATGATTTT TTAAATTTG ATGACAAGTT TGCATATTTA GTATCCAACA 1260

TACCCATTGA GCTTCTAGGA AATGTCAAGT CTATTAGAGA GAAAATTATA AAATGCTTCT 1320

CATCAGAAAA GTTAGCCAAG ATGCAAGGAT GTCAGAAGT TTTCAAAGC AGGCAAGATG 1380

ACCTGGAGAA ATATTATGTG CACGAGGACC TTGAAATAGG AGGTAAGAAC TTCTGAATGA 1440

30 GCACTTGCCT AAATAAAAAT CATTTACATA GACCTCTGAA ATAAAAAAG ACAAATGGC 1500

7	GACCTTGAAA ATTTTAT GCTCTTCTA ATTGGCTAAT GATAAATGTT TACTCTGATA	1560
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	TAATGAGAAA GCGTATAACT AAGCTGCATT TATCCCTCTT ATCTCATCCC CGACCACACC	1680
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	AAGCCTATTG GTTCTCATT GCATGCAGTG ATCCTTGGCT GGTCTGTGTC CTAACATCTT	1800
	TTAATTAGCA CACTGCAAAT CTAATCAGTG TAATAACGC TATTAATCTT CCTTTACACT	1860
	TATTTCTCC CACACATCAT TTAGGCTTTC TCTGGGCCTC TGTGGCAAAC ACTATTCCAA	1920
	CTATGTTCTG GGCAACGTAT TATCTTCTGC GGCACCCAGA AGCTATGGCA GCAGTGCCTG	1980
10	ACGAAATGA CCGTTTGCTG CAGTCAACAG GTCAAAGGA AGGGTCTGGA TTTCCCATCC	2040
	ACCTCACCAG AGAACAATTG GACAGCCTAA TCTGCCTAGG TAATTATTTT ATCTGTTATG	2100
	AAGAAAGAAG GTACCTCTCT GCAAACCTCG TTTATCACTC ATAGCTGTTT ACAAGAGGTA	2160
	GAGGACACAG CTGCTAATTG ACATAATAAC TCCCATTTC ATCAATTATA AATTATGTAG	2220
	TTTATAGCCG TAGATCATCT CATTGCATGT AAACATAAGG CCTATGTAAT TAACTGTGTA	2280
15	ATGTATGTAA AATTCTAACC AAAGCTTNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2340
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2400
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2460
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2520
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2580
20	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2640
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2700
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2760
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2820
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN	2880
25	AAGTTAAATT CCATACCAAT GAGTTATTCT CT TCTC TGTATTGACA TTTCATCTGC	2940
	GGTATCCTTT AGGGTACAAT GAGTTATTCT CT TCTC TGTATTGACA TTTCATCTGC	3000
	GGTATCCTTT AGGGTACAAT ATTCCAAGTT TCTTTAGACA AACGCAGGAA CAAATGTTCA	3060
	CATATTTCTG TTTCTTTATT CCTTTGACAA GTAGGCGAGC ATTTTAGCCT ATGTTGGTCT	3120
	CAAAAAAAT CTTTAAATA TGTTCAGGT TCTTTAATGG GACCTTTCAG GAGCAAAAGT	3180

CCTCCCAGGT TTGGTCAATG TTCACCCTCN GTGGCCATTG AGGAAAATGC CCNNNNNGTT 3240
 CTAGAGATTG TTCTCACTTC TCAGGCTAAG GCCCATTGAG CAATGCCAGA AAGCATGCCT 3300
 TATACTAGCA GTCAATTTGG AAGTTTGTAG TTTGTGTCTT TAGCATAGGT TATCAAATAA 3360
 ATTTTATATT TNCTTTTAAA AAAATCTCAA CATTACTAAA ATACAAATAT CCTTTTATTT 3420
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 TCCACTTGAT ACACAGTATT TCTGCATATT GTAATTTCTA TGAAGATCTA GGTTCATTT 3540
 CCCATACATT CAAGCAGTTT CCATTGCATT TTTATGAATA AGATGACGCA TACTGGGAAG 3600
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 AAAGTAGTTG TAATTCATCC ACTCTTTTAA CTTTCAACTT TTTGCTATTA AAAAATCATT 3720
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 TCTAGAACTA TAATTTGAAT CCATTTAAGC CCATAGCTAG AGCTAGAGAT TTTCACTATT 3840
 GGATCC 3846

(2) INFORMATION FOR SEQ ID NO: 4:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 415 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

20 Ala Leu Glu Tyr Gln Tyr Val Met Lys Asn Pro Lys Gln Leu Ser Phe
 1 5 10 15
 Glu Lys Phe Ser Arg Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys
 20 25 30
 25 Leu Leu Thr Asn Asp Asp Leu Ser Asn Asp Ile His Arg Gly Tyr Leu
 35 40 45
 Leu Leu Gln Gly Lys Ser Leu Asp Gly Leu Leu Glu Thr Met Ile Gln
 50 55 60
 Glu Val Lys Glu Ile Phe Glu Ser Arg Leu Leu Lys Leu Thr Asp Trp
 65 70 75 80
 30 Asn Thr Ala Arg Val Phe Asp Phe Cys Ser Ser Leu Val Phe Glu Ile
 85 90 95
 Thr Phe Thr Thr Ile Tyr Gly Lys Ile Leu Ala Ala Asn Lys Lys Gln
 100 105 110
 Ile Ile Ser Glu Leu Arg Asp Asp Phe Leu Lys Phe Asp Asp His Phe

	115	120	125
	Pro Tyr Leu Val Ser Asp Ile	Pro Ile Gln Leu Leu Arg Asn Ala Glu	
	130	135	140
5	Phe Met Gln Lys Lys Ile Ile Lys Cys Leu Thr Pro Glu Lys Val Ala		
	145	150	155
	Gln Met Gln Arg Arg Ser Glu Ile Val Gln Glu Arg Gln Glu Met Leu		
		165	170
	Lys Lys Tyr Tyr Gly His Glu Glu Phe Glu Ile Gly Ala His His Leu		
		180	185
10	Gly Leu Leu Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala Met Phe Trp		
		195	200
	Ala Met Tyr Tyr Leu Leu Gln His Pro Glu Ala Met Glu Val Leu Arg		
		210	215
15	Asp Glu Ile Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro		
		225	230
	Gly Ile Ser Val His Phe Thr Arg Glu Gln Leu Asp Ser Leu Val Cys		
		245	250
	Leu Glu Ser Ala Ile Leu Glu Val Leu Arg Leu Cys Ser Tyr Ser Ser		
		260	265
20	Ile Ile Arg Glu Val Gln Glu Asp Met Asp Phe Ser Ser Glu Ser Arg		
		275	280
	Ser Tyr Arg Leu Arg Lys Gly Asp Phe Val Ala Val Phe Pro Pro Met		
		290	295
25	Ile His Asn Asp Pro Glu Val Phe Asp Ala Pro Lys Asp Phe Arg Phe		
		305	310
	Asp Arg Phe Val Glu Asp Gly Lys Lys Lys Thr Thr Phe Phe Lys Gly		
		325	330
	Gly Lys Lys Leu Lys Ser Tyr Ile Ile Pro Phe Gly Leu Gly Thr Ser		
		340	345
30	Lys Cys Pro Gly Arg Tyr Phe Ala Ile Asn Glu Met Lys Leu Leu Val		
		355	360
	Ile Ile Leu Leu Thr Tyr Phe Asp Leu Glu Val Ile Asp Thr Lys Pro		
		370	375
35	Ile Gly Leu Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp		
		385	390
	Ser Asp Ile Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg Ser *		
		405	410

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 508 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

Met Gln Gly Ala Thr Thr Leu Asp Ala Ala Ser Pro Gly Pro Leu Ala
 1             5             10             15
10 Leu Leu Gly Leu Leu Phe Ala Ala Thr Leu Leu Leu Ser Ala Leu Phe
    20             25             30
    Leu Leu Thr Arg Arg Thr Arg Arg Pro Arg Glu Pro Pro Leu Ile Lys
        35             40             45
15 Gly Trp Leu Pro Tyr Leu Gly Met Ala Leu Lys Phe Phe Lys Asp Pro
    50             55             60
    Leu Thr Phe Leu Lys Thr Leu Gln Arg Gln His Gly Asp Thr Phe Thr
    65             70             75             80
    Val Phe Leu Val Gly Lys Tyr Ile Thr Phe Val Leu Asn Pro Phe Gln
        85             90             95
20 Tyr Gln Tyr Val Thr Lys Asn Pro Lys Gln Leu Ser Phe Gln Lys Phe
    100            105            110
    Ser Ser Arg Leu Ser Ala Lys Ala Phe Ser Val Lys Lys Leu Leu Thr
        115            120            125
25 Asp Asp Asp Leu Asn Glu Asp Val His Arg Ala Tyr Leu Leu Leu Gln
    130            135            140
    Gly Lys Pro Leu Asp Ala Leu Leu Glu Thr Met Ile Gln Glu Val Lys
    145            150            155            160
    Glu Leu Phe Glu Ser Gln Leu Leu Lys Ile Thr Asp Trp Asn Thr Glu
        165            170            175
30 Arg Ile Phe Ala Phe Cys Gly Ser Leu Val Phe Glu Ile Thr Phe Ala
    180            185            190
    Thr Leu Tyr Gly Lys Ile Leu Ala Gly Asn Lys Lys Gln Ile Ile Ser
        195            200            205
35 Glu Leu Arg Asp Asp Phe Phe Lys Phe Asp Asp Met Phe Pro Tyr Leu
    210            215            220
    Val Ser Asp Ile Pro Ile Gln Leu Leu Arg Asn Glu Glu Ser Met Gln
    225            230            235            240
    Lys Lys Ile Ile Lys Cys Leu Thr Ser Glu Lys Val Ala Gln Met Gln

```

	245	250	255
	Gly Gln Ser Lys Ile Val Gln Glu Ser Gln Asp Leu Leu Lys Arg Tyr		
	260	265	270
5	Tyr Arg His Asp Asp Ser Glu Ile Gly Ala His His Leu Gly Phe Leu		
	275	280	285
	Trp Ala Ser Leu Ala Asn Thr Ile Pro Ala Met Phe Trp Ala Met Tyr		
	290	295	300
	Tyr Ile Leu Arg His Pro Glu Ala Met Glu Ala Leu Arg Asp Glu Ile		
	305	310	315
10	Asp Ser Phe Leu Gln Ser Thr Gly Gln Lys Lys Gly Pro Gly Ile Ser		
	325	330	335
	Val His Phe Thr Arg Glu Gln Leu Asp Ser Leu Val Cys Leu Glu Ser		
	340	345	350
15	Thr Ile Leu Glu Val Leu Arg Leu Cys Ser Tyr Ser Ser Ile Ile Arg		
	355	360	365
	Glu Val Gln Glu Asp Met Asn Leu Ser Leu Glu Ser Lys Ser Phe Ser		
	370	375	380
	Leu Arg Lys Gly Asp Phe Val Ala Leu Phe Pro Pro Leu Ile His Asn		
	385	390	395
20	Asp Pro Glu Ile Phe Asp Ala Pro Lys Glu Phe Arg Phe Asp Arg Phe		
	405	410	415
	Ile Glu Asp Gly Lys Lys Lys Ser Thr Phe Phe Lys Gly Gly Lys Arg		
	420	425	430
25	Leu Lys Thr Tyr Val Met Pro Phe Gly Leu Gly Thr Ser Lys Cys Pro		
	435	440	445
	Gly Arg Tyr Phe Ala Val Asn Glu Met Lys Leu Leu Leu Ile Glu Leu		
	450	455	460
	Leu Thr Tyr Phe Asp Leu Glu Ile Ile Asp Arg Lys Pro Ile Gly Leu		
	465	470	475
30	Asn His Ser Arg Met Phe Leu Gly Ile Gln His Pro Asp Ser Ala Val		
	485	490	495
	Ser Phe Arg Tyr Lys Ala Lys Ser Trp Arg Ser *		
	500	505	

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 266 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

10 Gly Lys Tyr Ile Thr Phe Ile Pro Gly Pro Phe Gln Tyr Gln Leu Val
 1 5 10 15

Ile Lys Asn His Lys Asn Leu Ser Phe Arg Val Ser Ser Asn Lys Leu
 20 25 30

Ser Glu Lys Ala Phe Ser Ile Ser Gln Leu Gln Lys Asn His Asp Met
 35 40 45

15 Asn Asp Glu Leu His Leu Cys Tyr Gln Phe Leu Gln Gly Lys Ser Leu
 50 55 60

Asp Ile Leu Leu Glu Ser Met Met Gln Asn Leu Lys Gln Val Phe Glu
 65 70 75 80

20 Pro Gln Leu Leu Lys Thr Thr Ser Trp Asp Thr Ala Glu Leu Tyr Pro
 85 90 95

Phe Cys Ser Ser Ile Ile Phe Glu Ile Thr Phe Thr Thr Ile Tyr Gly
 100 105 110

Lys Val Ile Val Cys Asp Asn Asn Lys Phe Ile Ser Glu Leu Arg Asp
 115 120 125

25 Asp Phe Leu Lys Phe Asp Asp Lys Phe Ala Tyr Leu Val Ser Asn Ile
 130 135 140

Pro Ile Glu Leu Leu Gly Asn Val Lys Ser Ile Arg Glu Lys Ile Ile
 145 150 155 160

30 Lys Cys Phe Ser Ser Glu Lys Leu Ala Lys Met Gln Gly Trp Ser Glu
 165 170 175

Val Phe Gln Ser Arg Gln Asp Asp Leu Glu Lys Tyr Tyr Val His Glu
 180 185 190

Asp Leu Glu Ile Gly Ala His His Phe Gly Phe Leu Trp Val Ser Val
 195 200 205

35 Ala Ser Thr Ile Pro Thr Met Phe Trp Ala Thr Tyr Tyr Leu Leu Arg
 210 215 220

His Pro Glu Ala Met Ala Ala Val Arg Asp Glu Ile Asp Arg Leu Leu
225 230 235 240

Gln Ser Thr Gly Gln Lys Glu Gly Ser Gly Phe Pro Ile His Leu Thr
245 250 255

5 Arg Glu Gln Leu Asp Ser Leu Ile Cys Leu
260 265

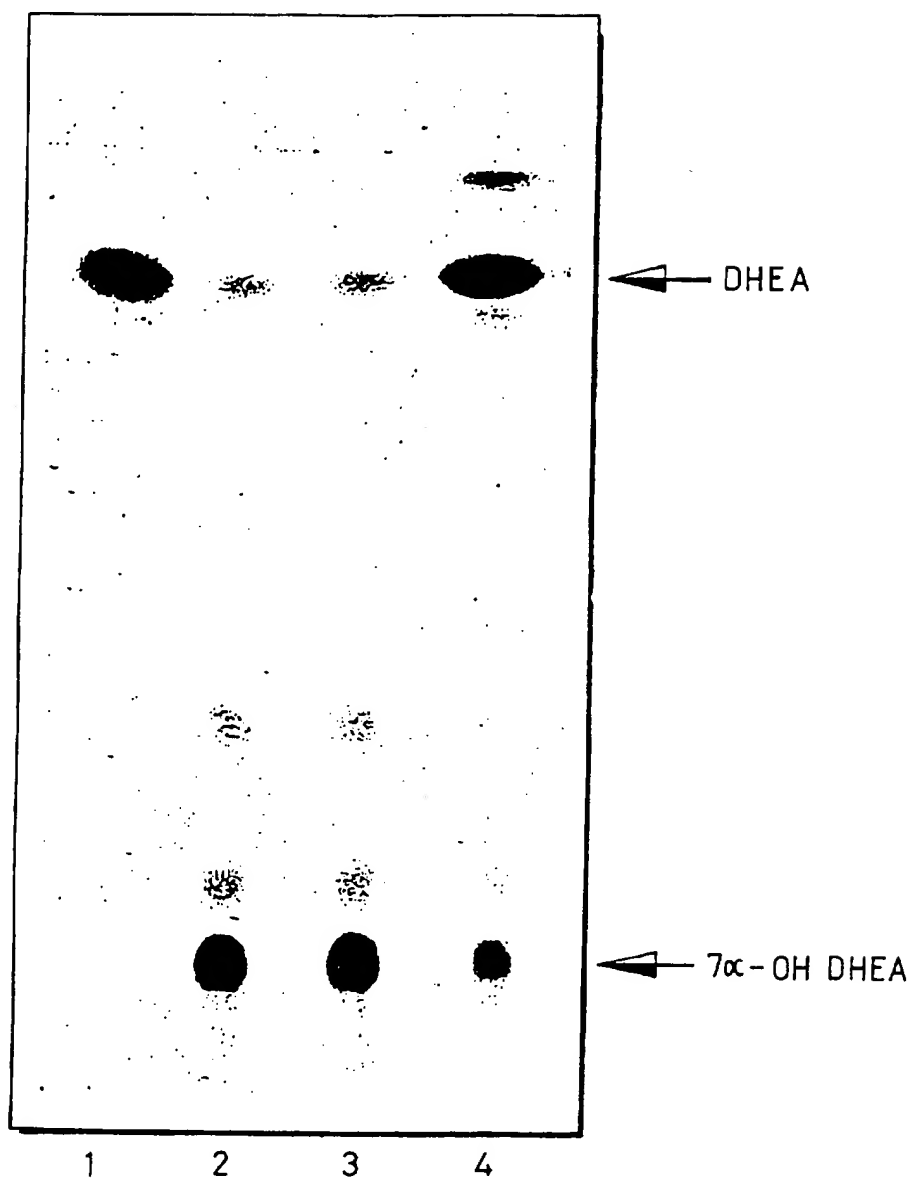


Fig. 1

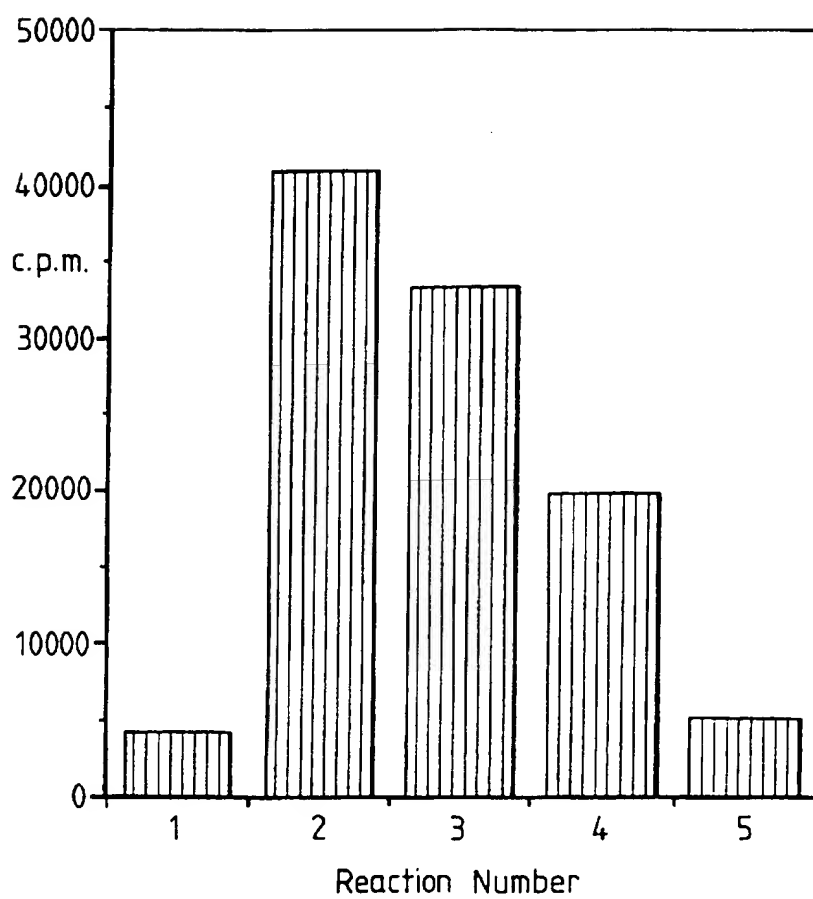
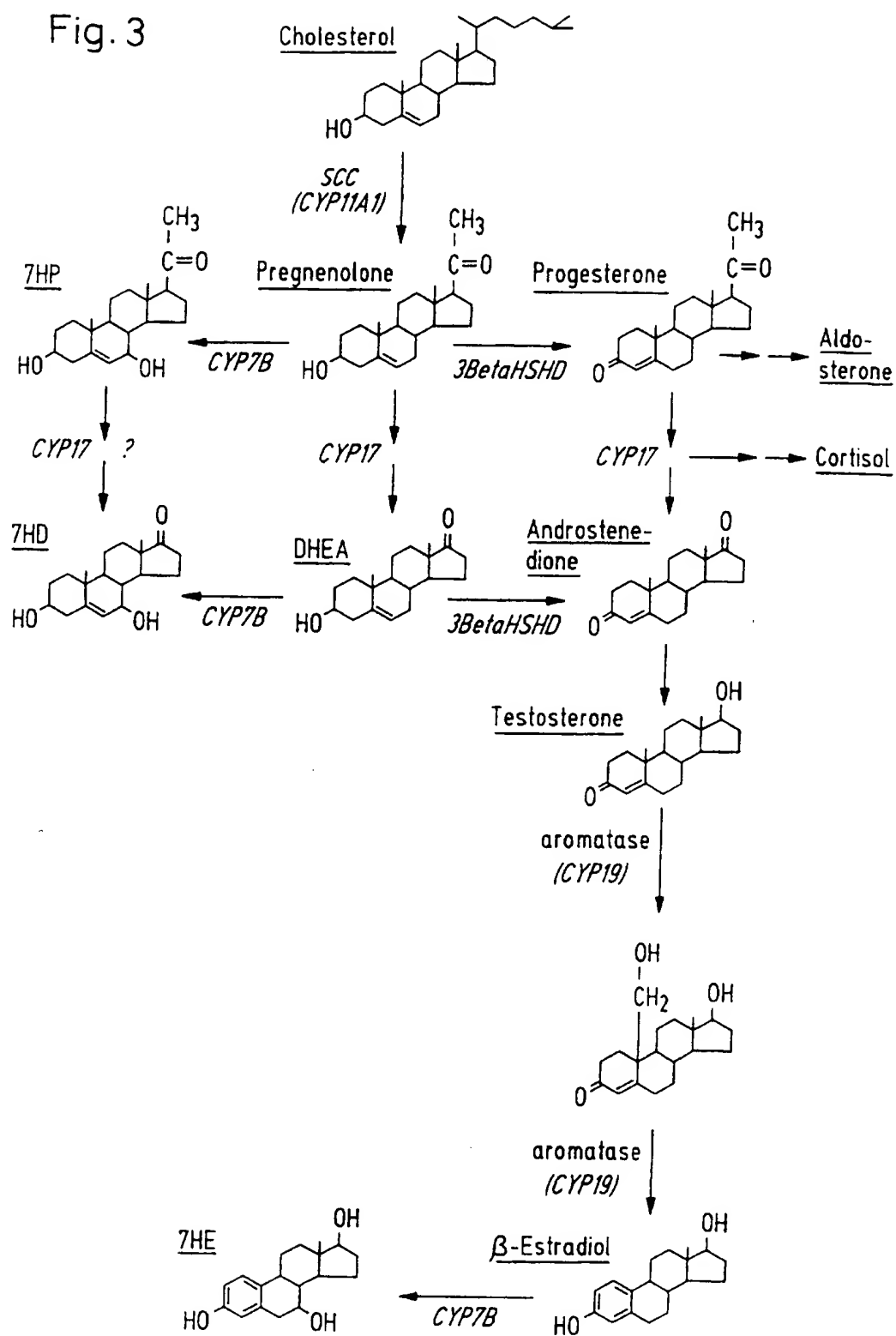


Fig. 2

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Fig. 3



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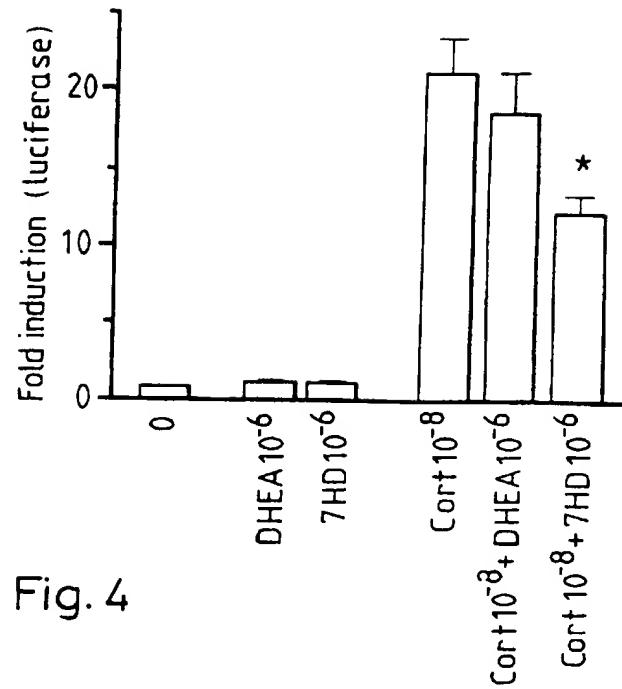


Fig. 4

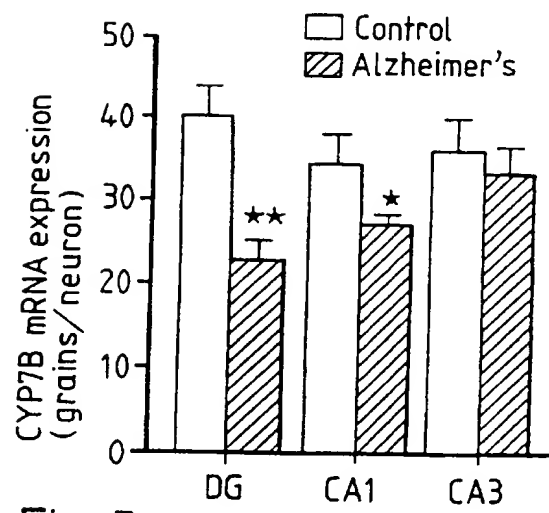
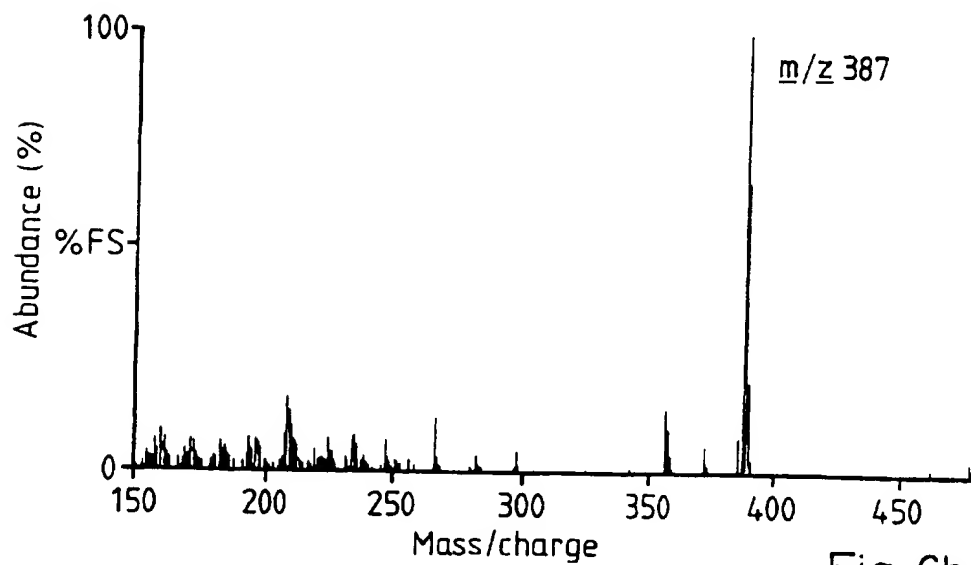
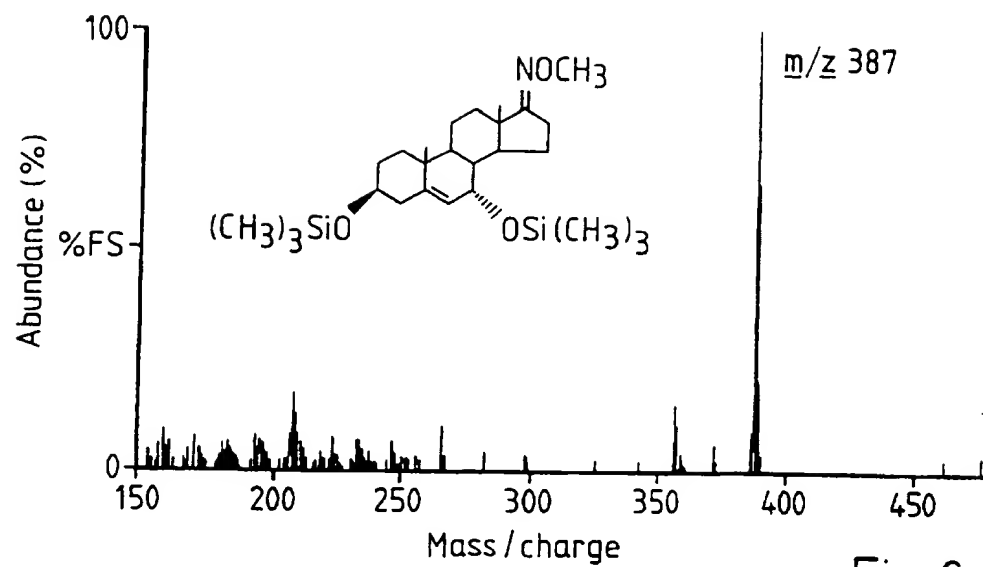


Fig. 5

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PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/565, C07J 1/00, C12P 33/00, A61K 39/395		A3	(11) International Publication Number: WO 97/37664 (43) International Publication Date: 16 October 1997 (16.10.97)									
(21) International Application Number: PCT/GB97/00955 (22) International Filing Date: 4 April 1997 (04.04.97) (30) Priority Data: <table border="0"> <tr> <td>9607289.7</td> <td>9 April 1996 (09.04.96)</td> <td>GB</td> </tr> <tr> <td>9608445.4</td> <td>24 April 1996 (24.04.96)</td> <td>GB</td> </tr> <tr> <td>9704905.0</td> <td>10 March 1997 (10.03.97)</td> <td>GB</td> </tr> </table> (71) Applicant (for all designated States except US): BRITISH TECHNOLOGY GROUP LTD. [GB/GB]; 101 Newington Causeway, London SE1 8BU (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): LATHE, Richard [GB/GB]; University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ (GB). ROSE, Kenneth, Andrew [GB/GB]; University of Edinburgh, West Mains Road, Edinburgh EH9 3JQ (GB). SECKL, Jonathan, Robert [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). BEST, Ruth [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). YAU, Joyce, Lai, Wah [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University		9607289.7	9 April 1996 (09.04.96)	GB	9608445.4	24 April 1996 (24.04.96)	GB	9704905.0	10 March 1997 (10.03.97)	GB	of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). LECKIE, Caroline, McKenzie [GB/GB]; Molecular Medicine Centre, Molecular Endocrinology, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU (GB). (74) Agent: DOLAN, Anthony, Patrick; British Technology Group Ltd., Patents Dept., 101 Newington Causeway, London SE1 8BU (GB). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 4 December 1997 (04.12.97)	
9607289.7	9 April 1996 (09.04.96)	GB										
9608445.4	24 April 1996 (24.04.96)	GB										
9704905.0	10 March 1997 (10.03.97)	GB										
(54) Title: USE OF 7 ALPHA-SUBSTITUTED STEROIDS TO TREAT NEUROPSYCHIATRIC, IMMUNE OR ENDOCRINE DISORDERS												
(57) Abstract Use is provided for a 7 α -hydroxy or 7-oxo substituted 3 β -hydroxy-steroid possessing the carbon skeleton of cholesterol, androsterone, pregnenolone or estradiol, or an analogue thereof substituted independently at one or both of the 7- and 3-positions with an ester or ether group, in the manufacture of a pharmaceutical composition for the therapy of neuropsychiatric, immune and/or endocrine disorders or for inducing cognitive enhancement. Uses for Cyp7b enzymes in producing such steroids is also provided together with various novel steroids and test kits and methods for diagnosing the disorders.												

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INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 97/00955

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/565 C07J1/00 C12P33/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07J C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 03176 A (HUMANETICS CORP.) 17 February 1994 see claims; examples ---	1-4, 28-33
P,X	WO 96 12810 A (UNIVERSITY OF EDINBURGH) 2 May 1996 cited in the application see claim 20 ---	7
P,X	K.A. ROSE ET AL.: "Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7alpha-hydroxy dehydroepiandrosterone and 7 alpha-hydroxy pregnenolone." BIOCHEMISTRY, vol. 94, no. 10, 1997, pages 4925-4930, XP002042014 see the whole document ---	11,12, 18-21
-/--		



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

29 September 1997

Date of mailing of the international search report

17.10.97

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Klaver, T

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/00955

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	K.A. ROSE ET AL.: "Steroid modification in brain: hydroxylation of pregnenolone and DHEA by the novel cytochrome P450, Cyp7b." J. ENDOCRINOL., vol. 152, 1997, page P280 XP002042015 see the whole document	11,12, 18-21
A	--- G. STAPLETON ET AL.: "A novel cytochrome P450 expressed primarily in brain." J. BIOL. CHEM., vol. 270, no. 50, 1995, pages 29739-29745, XP002042016 see the whole document	
A	--- EP 0 648 842 A (NORTHEASTERN OHIO UNIVERSITIES) 19 April 1995 -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 97/00955

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 28
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
It is not clear which compounds are described by "...or a derivative thereof..." nor is it clear which diseases are meant by descriptions like "...neuro-psychiatric, immune and/or endocrine disorders...". The search has therefore been limited to the examples mentioned in the claims and/or description.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☒ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 97 00955

FURTHER INFORMATION CONTINUED FROM PCT/ISA210

- 1) Claims 1-4, 28-33: Use of 7-substituted 3 β -hydroxy steroids and novel steroids, for the treatment of neuropsychiatric, immune and/or endocrine disorders.
- 2) Claims 5 - 2 : Use of Cyp7b to manufacture assay kits, to produce 7-hydroxy steroids, antibodies, and targeted drugs for gene therapy.
- 3) Claims 23 - 27 : Novel steroids of formula 1a and 1b.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. Application No

PCT/GB 97/00955

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9403176 A	17-02-94	US 5292730 A	08-03-94
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		EP 0746322 A	11-12-96
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EP 648842 A	19-04-95	US 5420028 A	30-05-95
		EP 0648840 A	19-04-95
		JP 7284393 A	31-10-95
		JP 7284388 A	31-10-95
